Triage of HPV-positive women by methylation marker analysis

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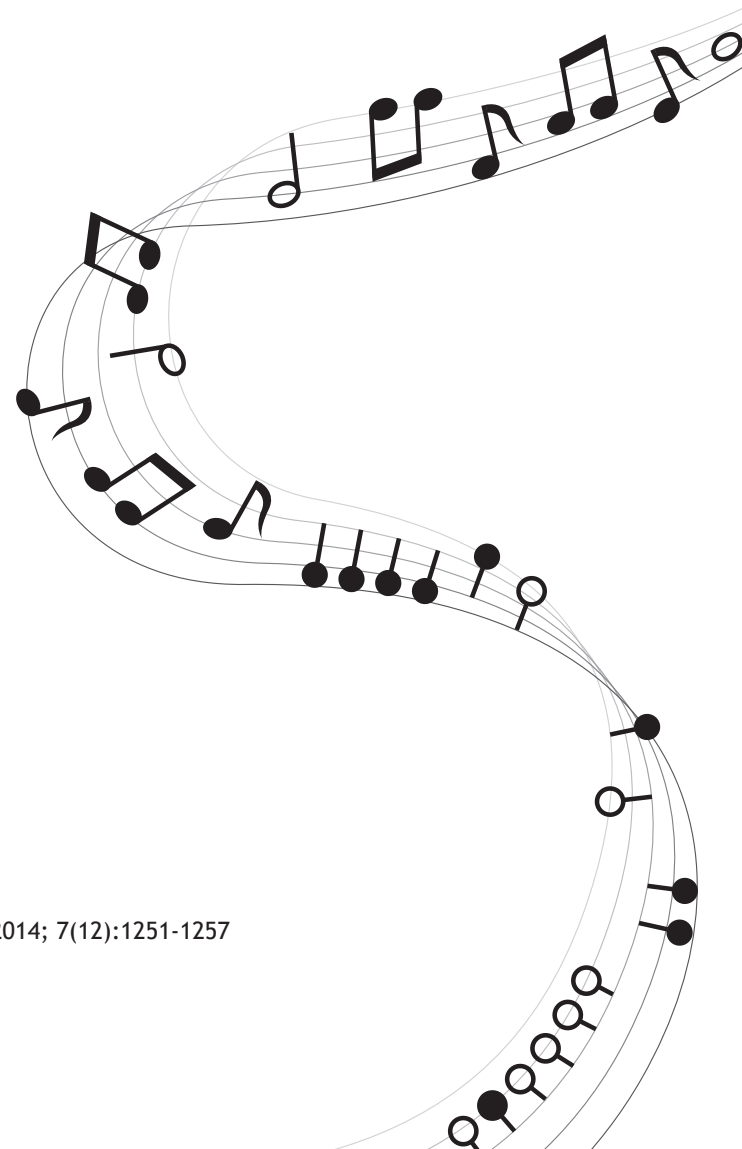
Chapter 5

Methylation analysis of the *FAM19A4* gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions



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ABSTRACT

Primary testing for human papillomavirus (HPV) in cervical screening requires triage to differentiate women with transient infection from those with persistent infection who require more intensive management given their risk for cervical (pre)cancer. In this study, the clinical performance of a novel methylation marker *FAM19A4* for the triage of high-risk (hr) HPV-positive women was evaluated. Using a training-validation set approach, we analysed a *FAM19A4* quantitative methylation-specific PCR (qMSP). The training set comprised hrHPV-positive cervical scrapes of 43 women with cervical intraepithelial neoplasia grade 3 or worse (CIN3+) and 135 women with ≤CIN1. The validation set comprised hrHPV-positive cervical scrapes of 52 women with CIN2+, including 33 CIN3+, 19 CIN2, and 166 women with ≤CIN1. The methylation threshold of *FAM19A4* qMSP that gave rise to CIN3+ specificity of 70% in the training set was applied in the validation set. This resulted in CIN3+ sensitivity of 75.8% (95% CI: 61.1-90.4) at 67.0% (95% CI: 60.3-73.8) specificity. Next, the validated qMSP was applied to an independent series of hrHPV-positive cervical scrapes of 22 women with cervical cancer, 29 with advanced CIN2/3 (i.e. women with a known preceding hrHPV infection (PHI) lasting ≥5 years as proxy of longer duration of lesion existence), and 19 with early CIN2/3 (i.e. PHI <5 years). All carcinomas (22/22) and advanced CIN2/3 lesions (29/29) were *FAM19A4* methylation-positive, compared to 42.1% (8/19; 95% CI: 19.9-64.3) of early CIN2/3 lesions. In conclusion, *FAM19A4* is an attractive triage marker for hrHPV-positive women, with a high reassurance for the detection of cervical carcinomas and advanced CIN2/3 lesions.

INTRODUCTION

An infection with a high-risk type of human papillomavirus (hrHPV) is necessary, however, not sufficient for the development of cervical cancer[1]. Following a persistent hrHPV infection, genetic and epigenetic changes in the host and/or viral genome are acknowledged to be involved in progression towards invasive cervical cancer[2,3]. Functional studies have shown that gene silencing by promoter hypermethylation of some tumour suppressor genes is a contributing factor to cervical carcinogenesis[4-8]. Gene promoter hypermethylation can be easily assessed by sensitive, (quantitative) methylation-specific PCR (MSP)-based methods on cervical scrapes and even self-collected cervico-vaginal samples. This has led to the idea that methylation analysis can provide an attractive early detection biomarker, amongst others, to be used as triage method for hrHPV-positive women in cervical screening[3,9]. Indeed, promising results have been obtained[10-15] with sensitivities for CIN2+ (i.e. cervical intraepithelial neoplasia (CIN) grade 2 (CIN2), CIN3 and cervical cancer) and CIN3+ (i.e. CIN3 and cervical cancer) similar to those of cytology analysis on cervical scrapes[11,12,] the latter currently being the most widely suggested triage tool. Of interest, recent work has revealed that methylation levels of several genes are particularly high in cervical scrapes of women with cervical cancer and advanced high-grade CIN lesions, the latter characterized by a longer duration (≥5 years) of a preceding hrHPV infection (PHI)[3,16]. As a consequence, methylation analysis could be particularly effective in detecting advanced precursor lesions (with likely a high short-term progression risk) and cervical cancers[3], and can serve as a complementary tool for cytology to gain a higher reassurance of not missing advanced lesions[17].

Most studies performed so far have used panels of methylation markers to reach sufficiently high sensitivities for high-grade CIN and cervical cancer[11,13,18,19]. In search for novel methylation markers, we recently have performed methylation-specific digital karyotyping of different passages of HPV16E6E7-transduced primary human foreskin keratinocytes[20]. This study resulted in the identification of novel DNA methylation events, including some directly following HPV16E6E7 expression, and others associated with the acquisition of an immortal phenotype (i.e. representing disease progression). The latter involved *FAM19A4, LHX1, NKX28, PHACTR3* and *PRDM14* genes. Pilot studies identified *FAM19A4* as a promising candidate methylation triage marker for hrHPV-positive women. *FAM19A4* (family with sequence similarity 19 (chemokine (C-C motif)-like), member A4) is a member of the TAFA family of five highly homologous genes that encode small secreted proteins. These proteins contain conserved cysteine residues at fixed positions, and are distantly related to MIP-1alpha, a member of C-C chemokine family that can serve as immunoregulator and chemokine[21].

The present study describes the verification and validation of the clinical performance of *FAM19A4* methylation analysis by qMSP in a large series of hrHPV-positive cervical scrapes derived from a screening population. The validated *FAM19A4* qMSP assay was additionally evaluated in an independent series of hrHPV-positive cervical scrapes in relation to severity and duration of the underlying lesion. For this purpose, scrapes from women with cervical cancer, and women with CIN2/3 with a PHI of <5 years or ≥5 years, were used. PHI was used as proxy of lesion duration, and accordingly these CIN2/3 lesions were assigned as early and advanced disease stages[16] respectively.

MATERIALS AND METHODS

Study population

Cervical scrapes for training and validation

Independent training and validation sets of hrHPV-positive cervical scrapes (n=178 and n=218, respectively) were used. For the training set, baseline cervical scrapes of hrHPV-positive women who participated in the intervention arm of a population-based cervical screening trial (POBASCAM)[22,23] were used. Scrapes in the validation set were from women who participated in population-based screening using the same screening and referral algorithm as in the intervention arm of the POBASCAM trial[11,24]. For all women, co-testing for hrHPV and cytology on the cervical scrapes at baseline was performed. Cytology was scored using the CISOE-A classification which is standard in the Netherlands and can be translated into the Bethesda classification[25]. In this classification, borderline or mild dyskaryosis (BMD) equals low-grade squamous intraepithelial lesions (LSIL), atypical squamous cells of undetermined significance (ASCUS) or atypical squamous cells not excluding high-grade squamous intraepithelial lesions (ASC-H). Moderate or worse dyskaryosis (>BMD) equals high-grade squamous intraepithelial lesions (HSIL). All women with baseline >BMD cytology were directly referred for colposcopy, independent of hrHPV status. All hrHPV-positive women with baseline BMD cytology were advised to repeat cytology and hrHPV testing 6 and 18 months later. They were referred for colposcopy at 6 months, in case of >BMD cytology, or BMD cytology combined with a hrHPV-positive test result, and at 18 months in case of >BMD cytology and/or a hrHPV-positive test result. Women with a positive hrHPV test result and normal cytology at baseline were referred to repeat cytology and hrHPV testing at 6 and 18 months. They were referred to colposcopy at 6 months in case of >BMD cytology, and at 18 months in case of >BMD cytology and/or a positive hrHPV test result. At colposcopy visit, biopsies were taken for histology according to standard procedures in the Netherlands[26].

The training set comprised hrHPV-positive scrapes of 178 women. Of these, 43 were of women that were histologically diagnosed with a CIN3+ lesion within 36 months of follow-up (including 4 squamous cell carcinomas (SCCs) and 1 adenocarcinoma (AdCA)).

These women had a median age of 31 years (range: 25-55) and 15 women had normal and 28 abnormal (i.e. BMD or worse) cytology at baseline. The remaining 135 scrapes were of women without evidence of CIN2+ further referred to as ≤CIN1 (including 27 CIN1 and 9 histologically-confirmed absence of CIN) up to the next screening round (5 years later) and had a median age of 34 years (range: 17-61). Of these, 99 women had normal and 36 abnormal cytology at baseline. The training set did not include any CIN2 lesions as CIN3+ is a better end-point for (pre)cancer[11,27].

The validation set comprised a consecutive series of 250 hrHPV-positive scrapes. Of 218 samples, sufficient material was left for qMSP analysis and valid qMSP results were obtained. This series comprised 52 women with a CIN2+ lesion within 36 months of follow-up (2 SCCs and 1 adenocarcinoma in situ (ACIS), 30 CIN3, 19 CIN2) with a median age of 34.5 years (range: 24-58). Of these, 19 women had normal and 33 abnormal cytology at baseline. The remaining 166 scrapes were of women who had no evidence of CIN2+ (including 11 CIN1 and 8 histologically-confirmed absence of CIN) within the same follow-up time. Of these, 139 had normal and 27 had abnormal cytology at baseline. The median age of this group was 39 years (range: 19-62).

Cervical scrapes of women with cervical carcinomas or CIN2/3 with a known duration of preceding hrHPV infection

Separate series of hrHPV-positive cervical scrapes were used to quantitatively evaluate *FAM19A4* methylation in relation to severity and duration of the underlying lesion. This series comprised (i) 22 hrHPV-positive cervical scrapes of women diagnosed with cervical cancer during population-based screening or whilst visiting a gynaecological outpatient clinic (19 SCCs, 1 adenosquamous carcinoma and 2 AdCAs). Of these scrapes, 19 had abnormal and 3 normal cytology. The median age of women was 38 years (range: 30-85); and (ii) 48 hrHPV-positive cervical scrapes of women diagnosed with a CIN2/3 in the second round of the control arm of the POBASCAM trial (blind HPV testing), and accordingly have a known 5-year history of hrHPV infection. The scrapes evaluated preceded the CIN2/3 biopsy. The duration of prior hrHPV infection was considered a proxy for duration of CIN2/3 existence[16,28]. Women with same hrHPV-type in both screening rounds were considered to have a PHI of ≥5 years, and their CIN2/3 lesions were considered advanced CIN2/3 lesions (n=29). Women who acquired the hrHPV infection after study entrance (PHI<5 years) were considered to have early CIN2/3 lesions (n=19). Of the 29 hrHPV-positive scrapes of women with advanced CIN2/3, 5 had normal cytology and 24 had abnormal cytology. The median age of these women was 40 years (range: 34-56). Of the 19 hrHPV-positive scrapes of women with early CIN2/3, 7 had normal cytology and 12 had abnormal cytology. The median age of these women was 40 years (range: 39-50).This study followed the ethical guidelines of the Institutional Review Board of the Medical Center.

DNA isolation, bisulphite treatment, and qMSP methylation analysis

DNA from cervical scrapes was isolated using the Nucleo-Spin 96 Tissue kit (Macherey-Nagel) and a Microlab Star robotic system (Hamilton, Germany) according to manufacturers' protocol[11]. Extracted DNA was subjected to bisulphite treatment using the EZ DNA Methylation Kit (Zymo Research, USA) as described previously[7,8]. Bisulphite-converted DNA was used as template for DNA methylation analysis. DNA methylation analysis of *FAM19A4* was performed by qMSP using housekeeping gene ß-actin (*ACTB*) as a reference gene[20]. A multiplex qMSP assay was developed according to criteria described by Snellenberg et al.[29]. Specificity of each primer pair for bisulphite-converted methylated DNA was confirmed by absence of amplification of unmodified DNA to ensure that no amplification would occur in case of incomplete bisulphite conversion. Quantification Cycle (Cq) values were measured at a fixed fluorescence threshold. Samples with a Cq>40 for *FAM19A4* were considered to represent a negative test result. All samples had a Cq value for *ACTB*<32 to assure good sample quality. All analyses were performed on an ABI 7500 real-time PCR-system (Applied Biosystems, USA). The *FAM19A4* result of a sample was expressed in Cq ratio, calculated by the following formula: $2^{[Cq\ (ACTB)\ -\ Cq\ (FAM19A4)]}$ x100.

Statistical analysis

Assessment of *FAM19A4* methylation analysis was performed by a training-validation set approach using two independent series of cervical scrapes that were sufficiently large to ensure an unbiased assay analysis. In the training set, a Receiver Operating Characteristics (ROC) curve of the *FAM19A4* qMSP assay was made for all ratio values and the area under the curve (AUC) was determined. The threshold value that gave rise to a CIN3+ specificity of 70% in the training set was chosen to consider a specimen positive for *FAM19A4* methylation. With this threshold, the biomarker test was converted into a categorical variable and subsequently evaluated in the independent validation set. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and referral rate were determined together with 95% Wald confidence intervals (95% CI), for outcome CIN2+ and CIN3+. The threshold value for scoring cytology positive was ASCUS (i.e. BMD). For quantitative evaluation of *FAM19A4* methylation, fold changes over a reference category (i.e. hrHPV-positive women with ≤CIN1 in the validation set) were determined. Differences in methylation levels between the different groups were analysed by Mann–Whitney U test. P-values below 0.05 were considered significant. All statistical analyses and computation of graphs were performed in IBM SPSS Statistics 20 and Excel.

RESULTS

Clinical validation of *FAM19A4* on physician-taken cervical scrapes of hrHPV-positive women

In the training set of 178 hrHPV-positive cervical scrapes, *FAM19A4* methylation analysis revealed a significant discrimination of women with CIN3+ from those with ≤CIN1 (p=0.004). ROC curve analysis showed an AUC of 0.775 (Figure 1). The methylation threshold of *FAM19A4* that in the training set gave rise to a CIN3+ specificity of 70% was chosen for validation set analysis. In the validation set (n=218), application of this threshold resulted in a CIN3+ sensitivity of 75.8% (95% CI: 61.1-90.4) at a specificity of 67.0% (95% CI: 60.3-73.8), and a CIN2+ sensitivity of 69.2% (95% CI: 56.7-81.8) at 69.9% (95% CI: 62.9-76.9) specificity. In comparison, application of cytology (threshold ASCUS) in this set, reached a CIN3+ sensitivity of 63.6% (95% CI: 47.2-80.0) at a specificity of 78.9% (95% CI: 73.0-84.4), and a CIN2+ sensitivity of 63.5% (95% CI: 50.4-76.5) at 83.7% (95% CI: 79.1-89.3) specificity. Corresponding PPVs, NPVs, and referral rates for endpoints CIN2+ and CIN3+ are presented in Table 1. It was observed that *FAM19A4* methylation analysis scored more carcinomas and CIN3 lesions positive than cytology [i.e. 3 carcinomas by *FAM19A4* compared to 2 by cytology, and 73.3% CIN3 (95% CI: 57.5-89.2) by *FAM19A4* compared to 63.3% CIN3 (95% CI: 46.1-80.6) by cytology]; while cytology was more often positive among CIN2 lesions [i.e. 57.9% (95% CI: 35.7-80.1) by *FAM19A4* compared to 63.2% (95% CI: 41.1-84.4) by cytology].

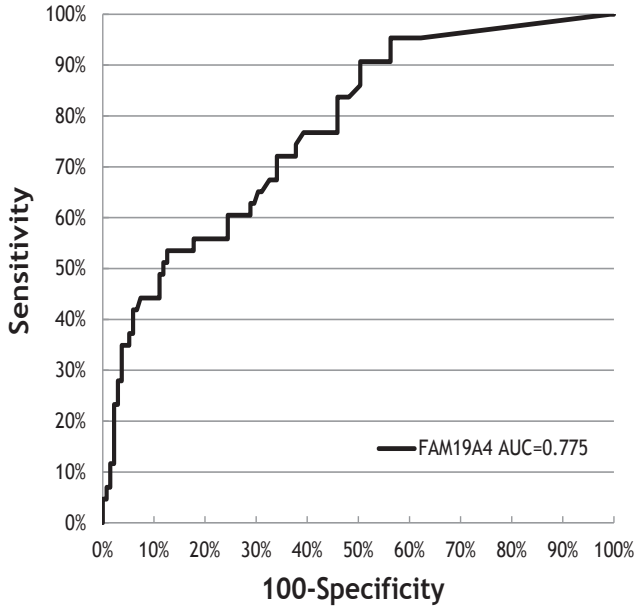

Figure 1. ROC curve of *FAM19A4* methylation analysis in the training set of 178 hrHPV-positive cervical scrapes. The sensitivity (y-axis) in relation to 1-specificity (x-axis) of *FAM19A4* methylation analysis is shown.

Table 1. Sensitivity, specificity, PPV, NPV, and referral rates for colposcopy for endpoints CIN2+ and CIN3+ in the validation set of 218 hrHPV-positive cervical scrapes.

Triage	Endpoint	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	Referral rate % (95% CI)
FAM19A4 [a]	CIN2+	69.2 (56.7-81.8)	69.9 (62.9-76.9)	41.9 (31.4-52.3)	87.9 (82.3-93.4)	39.4 (33.0-45.9)
	CIN3+	75.8 (61.1-90.4)	67.0 (60.3-73.8)	29.1 (19.5-38.7)	93.9 (89.9-98.0)	39.4 (33.0-45.9)
Cytology[b]	CIN2+	63.5 (50.4-76.5)	83.7 (79.1-89.3)	55.0 (42.4-67.6)	88.0 (82.9-93.0)	27.5 (21.6-33.5)
	CIN3+	63.6 (47.2-80.0)	78.9 (73.0-84.4)	35.0 (22.9-47.1)	92.4 (88.3-96.5)	27.5 (21.6-33.5)

[a] at the threshold that gave rise to a 70% CIN3+ specificity in the training set. [b] threshold ASCUS

FAM19A4 methylation in cervical scrapes of women with cervical cancer and CIN2/3 lesions with different duration of existence

We next evaluated the frequency of *FAM19A4* methylation positivity in an independent series of hrHPV-positive cervical scrapes of women with different underlying disease severities (i.e. early CIN2/3 with PHI<5 years (n=19), advanced CIN2/3 with PHI≥5 years (n=29) and cervical carcinomas (n=22)). *FAM19A4* methylation was particularly associated with advanced disease, scoring 100% positive in samples of women with cervical cancer (22/22) and women with advanced CIN2/3 lesions (29/29), compared to 42.1% (8/19; 95% CI: 19.9-64.3) of women with early CIN2/3 lesions (Figure 2). In the same series, cytology was abnormal (≥BMD) in 86.4% (19/22; 95% CI: 72.0-100) of women with cervical cancer, 82.8% (24/29; 95% CI: 69.0-76.5) of women with advanced CIN2/3 and 63.2% (12/19; 95% CI: 41.5-84.8) of women with early CIN2/3. Thus, *FAM19A4* methylation analysis tended to be more competent than cytology in detecting cervical carcinomas and advanced CIN2/3, whereas cytology had a relatively higher preference for early CIN2/3 (Figure 2). When considering *FAM19A4* methylation levels (expressed as Cq ratio; Table 2), an increase proportional to the duration of lesion existence was observed with both a significant increase between early and late CIN2/3 (p<0.001), and between late CIN2/3 and cervical cancer (p=0.001). A 3.1-fold increase in methylation levels is seen in early CIN2/3 compared to the reference (≤CIN1 validation set, n=166), elevating to 67.9 fold in advanced CIN2/3, and ultimately reaching highest levels in cervical cancer (270.8 fold).

Table 2. *FAM19A4* methylation levels per lesion category.

	Cq ratio *FAM19A4*		
category	**median**	**range**	**fold changes over reference**
≤CIN1[a]	0.123	0-41.94	1 (reference)
early CIN2/3	0.383	0-2.09	3.1
advanced CIN2/3	8.357	0.44-94.81	67.9
carcinoma	33.309	1.35-167.56	270.8

[a] ≤CIN1 from validation set (n=166)

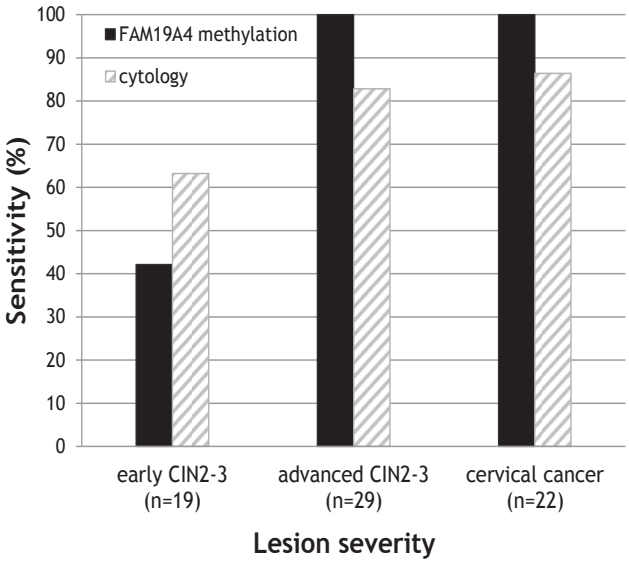

Figure 2. *FAM19A4* methylation analysis and cytology in relation to duration of CIN disease and cervical cancer. The sensitivity (y-axis) of *FAM19A4* methylation analysis (black bars) and cytology (grey bars, stripes) in hrHPV-positive cervical scrapes in relation to duration of CIN disease and cervical cancer (x-axis) is shown.

DISCUSSION

FAM19A4 Was previously identified by a genome-wide DNA methylation screen as a methylation event associated with the acquisition of an immortal phenotype of HPV16E6E7-transduced cells, and suggested as promising disease marker[20]. In this study, we verified the value of *FAM19A4* methylation analysis as triage marker to assess the presence of (pre)cancerous cervical lesions in hrHPV-positive women. We composed and validated the biomarker qMSP assay by a training-validation set approach, resulting in a triage marker for hrHPV-positive cervical scrapes that had an overall CIN3+ sensitivity of 75.8% (95% CI: 61.1-90.4) at 67.0% (95% CI: 60.3-73.8) specificity, and CIN2+ sensitivity of 69.2% (95% CI: 56.7-81.8) at 69.9% (95% CI: 62.9-76.9) specificity. Of note, the validated *FAM19A4* methylation assay detected all cervical carcinomas (22/22) and advanced CIN2/3 lesions (29/29). Consequently, the *FAM19A4* methylation marker can be a valuable tool to differentiate hrHPV-positive women who should be sent for colposcopy because of the presence of cancer or advanced CIN2/3 lesions with a high short-term progression risk for cancer, and therefore in need of treatment.

So far, methylation-based assays that have been put forward as triage tools for HPV-positive women make use of a panel of two to five markers to ensure sufficient detection of high-grade cervical lesions[11,13,18,19]. Here, we found that the *FAM19A4* marker alone had a similar performance in terms of CIN2+, CIN3+ and cervical carcinoma detection as a panel of previously studied methylation markers[11]. Recently, other single methylation markers, i.e. *PAX1* and *ZNF582* have been reported as promising methylation markers for cervical screening and women with LSIL, respectively[30,31]. Yet, these markers have not been validated in population-based screening studies and require further investigation.

CIN2/3 reflects a heterogeneous disease. Early and advanced CIN2/3 lesions, displaying a low and high short-term progression risk for cancer, respectively, can be distinguished on the basis of molecular host cell alterations[3]. Our study strengthens previous findings of DNA methylation analysis being more sensitive over cytology for the detection of the most advanced lesions and cervical cancers[3,16,17]. As shown in Figure 2, our findings are in line with a recent hypothesis that both tests do not detect exactly the same lesions; with DNA methylation analysis having a preference for detecting the more advanced CIN3+ lesions, and cytology tending to also detect early CIN2/3 lesions[3,17]. Our findings support that the *FAM19A4* methylation marker can serve as an alternative or complementary tool for cytology[17] to gain a higher reassurance of not missing advanced lesions and cervical cancer[3]. It should be noticed that our study was performed within the setting of well-organized screening in the Netherlands with a high quality standard of cytology reading[32]. In countries without less organized cytology infrastructure, objective molecular triage testing by the *FAM19A4* methylation marker might even have higher additive value in terms of reassurance.

In line with Bierkens et al.[16] reporting on *CADM1/MAL* methylation, methylation levels of *FAM19A4* increased with increasing disease severity, being particularly high in cervical scrapes of women with cervical cancer. These data reflect that hrHPV-positive women with a positive *FAM19A4* methylation test should be sent for immediate colposcopy given high risk of advanced lesions in need of treatment. *FAM19A4* methylation-negative women could be offered a repeat test after 12–18 months instead of direct colposcopy referral[3] which could markedly reduce over referral and overtreatment. The effect on patient outcome of such strategy, nonetheless, requires confirmation in a prospective trial with intervention based on methylation status. We acknowledge that in such scenario part of CIN2/3 lesions may remain undetected. These lesions are most likely early onset lesions with few chromosomal abnormalities[28], and are supposed to have low risk to progress to invasive cancer within the screening interval. Yet, to fully support this hypothesis, additional proof is needed, for example by demonstrating no or limited chromosomal aberrations in these lesions[28] or by active surveillance of women with *FAM19A4* methylation-negative, colposcopically evaluable small CIN2.

The high confidence of not having cervical cancer in case of absence of *FAM19A4* methylation, is supported by a recent analysis of an extra series of hrHPV-positive cervical scrapes of women with cervical SCC with unknown cytology (n=35), all of which were *FAM19A4* methylation-positive (data not shown).

Previous studies have shown that self-sampling can increase the participation rate in population-based screening and allows the efficient detection of CIN2/3+ lesions[33-38]. Since triage by methylation markers is possible on the same sample used for hrHPV-testing, this would obviate the need of additional visits to the physician for cytology triage[12]. Given recent studies on the application of methylation marker testing to self-collected specimens[10,12,15], further evaluation of *FAM19A4* as a methylation triage test in hrHPV-positive self-samples is warranted.

In conclusion, *FAM19A4* is an attractive methylation triage marker for hrHPV-positive women that reaches an overall CIN3+ sensitivity of 75.8% at a specificity of 67.0%, with particularly a high reassurance for the detection of cervical carcinomas and advanced CIN2/3 lesions.

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